1973

The Degradation of Octane in Continuous Culture

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THE DEGRADATION OF OCTANE IN CONTINUOUS CULTURE

A Thesis
Presented to the Graduate School of
the State University of New York
College at Brockport

As Partial Fulfillment of the
Requirement for the Degree of
Master of Science
in
Botany
by
Jui-Yu Chang
September, 1973

ACKNOWLEDGEMENT

I would like to express my sincere gratitude to my major advisor Dr. P. H. Pritchard for his suggesting this topic, advice and encouragement throughout this study.

I would also like to thank for the financial aid from the office of Naval Research, Grant No. N0014-73-C-0074.

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INTRODUCTION

There are many situations which lead to oil pollution of the aquatic environment. Some happen accidently, such as the Torrey Canyon shipping disaster and the Santa Barbara oil spill. Another source of continuous pollution results from oil-transportation and industrial wastes. The spreading of oil pollution has a more or less serious effect on the ecology of an aquatic environment and therefore must be removed.

Oil slicks in aquatic environments will disappear naturally by evaporation, auto-oxidation, and microbial degradation. Evaporation can remove some toxic portions of oil, but is limited to the hydrocarbons with low boiling points. Auto-oxidation is a slow process which is brought about by light and depends on the presence of certain factors, such as metal- or sulfur-containing compounds. It probably does not remove a significant part of an oily pollutant. Evaporation and auto-oxidation can not be controlled by man's activities and therefore they are not useful as a control of oil pollution.

Instead man has resorted to some means of mechanical or chemical treatment to clean up oil pollutants. The mechanical method can hardly remove the oil completely because the oil spreads out in a thin film over a large area. Under certain conditions the oil may also coalesce together with particular matter and sink making it virtually inaccessible. As for chemical means of cleaning up oil slicks the addition of detergents or emulsifiers to the oil surface only removes the oil from sight and does not necessarily remove it from the environment. Besides,

the chemical compounds added in some situations may be more toxic to the aquatic life than oil itself. Micrbial degradation of oil is the only adequate way to remove the oil pollutants because:

1) Bacteria can degrade very small concentrations of hydrocarbons, ultimately to CO_2.
2) Bacteria as a group have a wide range of substrate specificity.
3) Oil may be inaccessible to man but not to bacteria, which are always there and, given enough time, will degrade the oil.

In nature, the rate of oil degradation by bacteria is very slow. As such the danger of the pollution is increased because there is more chance for the oil to pollute our beaches and poison the plant and animal communities. Therefore, it is important to study the mechanism of hydrocarbon degradation by micro-organisms, with the purpose of trying to increase their effectiveness. Some attempts have been made to increase oil degradation rates. For example, by culturing hydrocarbon utilizing bacteria and seeding the most effective micro-organisms into the polluted area it may be possible to increase the oil degradation rates. Also, by providing the adequate circumstances for the bio-degradation process, a similar enhancement may be brought about.

The purpose of this study is to use the technique of continuous culture to study the microbial degradation of oil in aquatic environments.

Although much work has been done in the field of biodegradation of oil and hydrocarbons, very little of this work has used continuous culture techniques. The continuous system is an open system which will provide a condition more similar to the natural environment. It is the hope that the mechanisms of oil breakdown by bacteria in nature will be more understandable by the use of this technique.

In this study a normal alkane, octane, was chosen as the principle substrate to test the feasibility of the continuous culture approach. The reason octane was chosen was because: a) it is very slightly soluble in water b) its mechanism of degradation is known c) it is available in chemically pure form and d) it is relatively non-volatile.

Using mixed population and pure culture studies, we hoped to gain insight into how fast bacteria degrade hydrocarbons, to what degree the degradation rate is affected by environmental factors and whether the mixed populations of bacteria compete with each other during the degradation process.

Since hydrocarbons are poorly soluble in water, it has been thought that the hydrocarbons must be mechanically dispersed in the water phase in order to get microbial degradation. However, these dispersion techniques are difficult to incorporate into continuous culture systems. For example, large amounts of hydrocarbon are required, the amount of hydrocarbon dispersed must be constant with time and rubber tubing is required which will not be affected by the hydrocarbons.

To circumvent these problems, a continuous culture

system was designed in which the hydrocarbon is floated on the surface of a water column under conditions in which it was not dispersed. The purpose of such a device was to show in fact that the hydrocarbon could be degraded in this type of continuous culture system and to demonstrate the factors that affect the degradation process.

LITERATURE REVIEW

I. Oil and Hydrocarbon Degradation Studies

A) Organisms

The types of bacteria which grow at the expense of hydrocarbons and oil are quite numerous and their occurance has been well documented. The ability of bacteria to attack hydrocarbons was first noted by Söhngen in 1906. He found that methane was utilized by a bacillus, which he later named Methanomonas methanica. Söhngen (39) further reported that gasoline, kerosine, paraffin oil and paraffin was also could be oxidized to carbon dioxide, water and organic acids by bacteria isolated from soil and ditch water. These organisms studies by Söhngen belonged pricipally to the genera Mycobacterium and Pseudomonas. Bushwell and Hass (4) studied hydrocarbon utilization by various organisms and found that members of genera Micrococcus, Corynebacterium were also able to assimilate hydrocarbons. In a list compiled by Fühs (10) the number of genera harboring hydrocarbon utilizing species had grown to include Achromobacter, Mycobacterium and Nocardia, indicating that the ability to degrade hydrocarbon was a common attribute of the microbial world. This has been further emphasized by Foster (8) who pointed out that bacteria isolated from non-hydrocarbon media also had the ability to degrade hydrocarbons. This he stated was true of bacteria included in the genera of Alcaligenes, Bacillus, Brevibacterium, Corynebacterium, Flavobacterium, Micrococcus and Pseudomonas.

In a study by Perry et al (35) it was concluded that the variation in the capacity for hydrocarbon utilization was dependent on the nature of the substrate used for primary isolation. For example, about 10% of bacteria isolated on glucose and about 80% of organisms isolated on pentanone could use n-tridecane as sole carbon and energy source. Jones et al (20) took soil samples from different depths and identified a number of microflora from each sample which were responsible for hydrocarbon oxidations. In their study, bacteria belonging to the genera of Pseudomonas, Nocardia, Bacillus, and Mycobacterium were found at the depth of 5 cm, 20 cm, and 40 cm; Corynebacterium at the depth of 5 cm, 20 cm; and Arthrobacter and Micrococcus at the depth of 5 cm.

These and a number of other studies have indicated that utilization of oil and hydrocarbons as sources of carbon and energy is a characteristic common to many types of micro-organisms.

B) Environmental Conditions

Many different studies on oil and hydrocarbon degradation have shown the necessity for supplying sufficient quantities of nitrogen (either as ammonium or nitrate ions or amino acids), phosphorus and oxygen. These requirements are typical of other degradation studies. With hydrocarbons, however, oxygen is very critical because most of the mechanisms for initial attack on a hydrocarbon involve the direct insertion of molecular oxygen. Thus for a complex mixture of hydrocarbons, such as oil, oxygen supply may become the rate limiting step

in a degradation process. Hydrocarbons are usually very poorly degraded if at all under anaerobic conditions (44).

Several other conditions are also necessary to get good rates of degradation. For example other inorganic salts are sometimes required although in much smaller concentrations. A buffer is often necessary since the production of fatty acids from the hydrocarbons oxidation is very common and will often result in the lowering of the pH below growth supporting levels.

Since hydrocarbons are so poorly soluble in water, some form of dispersion or emulsification is generally employed in degradation studies. This is usually accomplished through the use of shake flask cultures but other more vigorous techniques are often used to get maximal degradation rates.

Microbial activity on hydrocarbons is also greatly influenced by temperature. The temperature at which bacteria are most active is between 20°C to 35°C. However, microbial degradation has been observed at low temperatures of about -2°C to high temperatures about 70°C. Klug and Markovetz(22) reported on the first thermophilic bacterium which utilized n-tetradecane at temperature of 45°C to 70°C. Atlas and Bartha (1) studied the biodegradation of petroleum in sea water at temperatures from 5°C to 20°C, and found that low temperatures retard biodegradation due to a retention of violatile inhibitors in crude oil.

C) Substrate Specificities

Foster (8) in his early studies indicated that any

one organism is capable of utilizing only a small portion of hydrocarbons and normal alkanes containing 10 to 18 carbons were good substrates for most organism. Johnson (18) showed that the range of alkanes attacked by different strains of bacteria varied. For example, one strain of Micrococcus grew on n-alkanes from C_8 to C_{11} whereas another strain grew on hydrocarbons from C_{12} to C_{20}. Thysse and Zwilling-deVries (40) studied iso-alkane oxidation by a strain of Pseudomonas aeruginosa and found that branched chain alkanes were hardly used by this bacteria and only compounds containing a single methyl substituent were attacked. McKenna (30) further showed that microbial assimilation of alkanes was affected by size, position, number of branches, as well as degree of branching of hydrocarbons. The above two studies supported the contention that branched chain alkanes were attacked with greater difficulty than n-alkanes and that a certain length of straight chain is required to render the substrate available. Perry (34) tested the ability of his isolates (pregrown on one hydrocarbon in the range of C_1 to C_8) to oxidize C_1 to C_8 normal paraffimic alkanes. His work demonstrated that bacteria usually oxidize the hydrocarbon of a chain length near or longer than the growth substrate. Lukins (28) found a high degree of substrate specificity in hydrocarbonoclastic bacteria. N-hexane, for example, can disrupt the integrity of the cytoplasmic membrane of one mycobacterium while serving as growth substrate for another mycobacterium in the same experimental conditions. The microbial degradation of hydrocarbons

was thus not only dependent on the configuration of the compound, but also on the bacterial species involved.

In the natural environment it appears that the normal alkanes are the first components of crude oil to be degraded. Jobson (19) for example, studied the degradation of crude oil by pure and mixed populations and found that the n-saturate fraction was preferentially attacked. This is probably a agreement with the statement by Zobell (44) that the n-alkanes are attacked by more microbial species, more rapidly and support more growth than other kinds of hydrocarbons.

II. Mechanisms of Alkane Degradation

Normal alkanes are generally metabolized by bacteria in two distinct steps. First they are oxidized by the insertion of molecular oxygen to form a fatty acid and then the fatty acid is further oxidized to CO_2 and cell biomass by β-oxidation. The mechanism of β-oxidation in bacteria appears to be universal in that no variations in the metabolism of fatty acids are readily recognized. However, the initial oxidation of the normal alkane appears to be somewhat more complicated and slightly more variable.

The production of a primary alcohol as the first step of alkane oxidation has been demonstrated in whole cell systems (38) as well as in cell free extracts (2).

The production of a primary alcohol from the initial oxidation of the alkane generally requires three proteins, iron, a reduced pyridine nucleotide (NADH or NADPH) and

oxygen (36). The three proteins which are responsible for conversion of octane to octanol in Pseudomonas oleovorans are hydroxylase, rubredoxin, and a diphosphopyridine nucleotide-rubredoxin reductase (36). The hydroxlase contains non-heme iron and is capable of introducing one atom of molecular oxygen into the alkane (29). The rubredoxin also contains non-heme iron and serves as an electron transport system for the hydroxylase. The rubredoxin-reductase catalyzes the transfer of electrons from NADH to rubredoxin (26). The primary alcohol formed in some species is further oxidized to aldehyde and fatty acid by the corresponding dehydrogenase (2).

Instead of a primary alcohol as the first product of alkane oxidation, an alkene is thought to be the initial intermediate in the oxidation. Chouteau et al (5) indicated that hept-1-ene was produced from n-heptane by Pseudomonas aeruginosa. Parekh et al (33) reported that an alkane dehydrogenase was found to reduce alkane to alkenes in a Pseudomonas strain. This kind of oxidation is not significant for alkane degradation since it has been difficult for workers to show further oxidation of alkene. Besides, most species used in this kind of study readily attack hydrocarbons via the hydroxylase system.

Besides C_1 as the target for oxidation of methyl groups, ω-oxidation is frequently encountered. Kester and Foster (21) reported on the production of dicarboxylic acids from C_{10} - C_{14} alkanes by a genus of Corynebacterium. Coon et al (2) also

reported on the ω-oxidation of octane by a soluble enzyme system.

Alkane oxidation has also been found to take place at a position other than terminal groups. Lukins and Foster (27), for example, showed the production of acetone, butan-2-one, pentan-2-one, and hexan-2-one from the respective alkanes by a genus of Mycobacterium. Fredricks (9) found the production of decan-2, 3, 4, and 5 ones together with the corresponding alcohols from a Pseudomonas aeruginosa grown on decane.

In some studies, a hydroperoxide has also been found as an metabolic intermediate (15, 24). The hydroperoxide formed is thought to be reduced to alcohol prior to acetone formation (24), or directly oxidized to ketone and aldehyde (22). The hydroperoxide oxidation appears to be a significant means of initial attack on hydrocarbons used by some bacteria which are not involved a typical hydroxylase system.

III. Work involving Octane Degradation

Octane has been used as a substrate for numerous hydrocarbon degradation studies and the conditions under which it is attacked by bacteria is well worked out. Jones et al (20) for example, found that a large number of bacteria from soil can grow on octane as the sole source of carbon and energy, and they include members of the genera Mycobacterium, Arthrobacter, Bacillus, Nocardia, and Pseudomonas. Gholson et al (11) showed the oxidation of octane was typical for all normal n-alkane oxidations, i.e. oxygen insertion to form an alcohol

followed by dehydrogenation to the respective fatty acid. Van Eyk et al (43)have shown in their work with a Pseudomonas species that octane is normally attacked through an inducible enzymatic system and this is probably the same situation found in most alkane oxidizers. Van Eyk and his group also found that the induction of an octane oxidation system also induced an enzyme system which allowed the cell to attack other n-alkanes as well. Evidence indicates that it is probably the same enzyme system responsible for the oxidation of hydrocarbon with different chain lengths.

IV. Batch culture techniques

The study of hydrocarbon degradation in the laboratory has classically employed the batch culture. This technique usually involves shake flask cultures in which organisms are shaken in a minimal salts medium containing a small quantity of hydrocarbon or oil. With this method, the substrate can be mechanically dispersed making it more available for microbial attack. This technique has been used for growth studies using pure cultures and enrichments for obtaining new hydrocarbon degrading isolates. The enrichment process consists of growing the cells in shake culture, continuous transfer of cell suspension to new medium after growth, and isolation of the predominant species.

Batch culture techniques, though very useful experimentally, are limited in their application to natural situations, because the environmental factors which affect the degradation

process, such as pH, oxygen concentration, availability of inorganic nutrients, production of metabolites and toxic substances, constantly vary with increased incubation time, i.e., they are time-dependent influences which are difficult to control (16). As a result, organisms growing in batch culture change physiologically and functionally to adapt to the continuous changes in batch culture. The succession of micro-organisms involved in hydrocarbon degradation will also be artifically controlled by these factors in closed system. Thus, the growth and succession of micro-organisms are a function of batch culture itself and not a function of the inherent limitations present in the natural environment (16).

V. Continuous culture techniques

Continuous culture techniques have recently been of importance to the study of microbial ecology (17). It is a continuous flow system in which individual cells are suspended in a constant volume, at or near a steady state of growth established by the continual addition of growth medium, and continued removal of part of the culture. The continuous culture system contains four basic parts: (1) a culture vessel (2) a nutrient supply system (3) a system for agitation of culture (4) a system for drainage that removes fluid from culture vessel (23).

The continuous culture techniques used in this study were based on continuous culture theory which has been well discussed by Monod (1952) and Herbert et al (1956) (14). It

is assumed that in continuous culture, the cell are growing in the exponential phase, and the population density is maintained at a fixed submaximal value. In a chemostat, all growth factors, except one, are in excess, and the environmental factors are kept constant. Therefore only one substance is the growth limiting factor. The concentration of organisms in culture is determined by the limiting substrate concentration and the growth rate of cells is equal to the dilution rate. Since growing cells are being washed from the growth vessel at a rate equal to their growth rate, the concentration of organism in the culture remains constant with time, and the concentration of growth limiting substrate in the culture does not change. The cell will be washed out when its maximum growth rate is smaller than the dilution rate.

The continuous culture technique offers several advantages over batch techniques in the study of natural system.

First, since one is dealing with an open system, the growing culture is constantly being diluted with fresh medium and at the same time the accumulation of toxic substances and metabolic products in the growth vessel is being prevented by the constant dilution (16).

Second, the environmental factors which vary indefinitely in batch culture, are time-independent in continuous culture system and are therefore more easily controlled (14).

Third, the bacterial population can be controlled by a single limiting factor or substrate concentration (14) The constant cell population at constant conditions makes the

study of cell physiology easier, because cell growth is controlled by a single limiting factor instead of many.

Fourth, it allows one to study the growth of bacteria at very low substrate concentraions. Since in nature, there are many bacterial species which would compete for the available nutrients, the use of a low concentration of limiting substrate in the chemostat allows the bacteria to grow in a condition similar to nature (41).

Jannasch(16) has isolated different bacteria from sea water by a chemostat enrichment technique using different dilution rates and different substrate concentrations as the basis for the selection process. In his experiments, fresh sea water was supplemented with a carbon source (lactate, glycerol or glucose), phosphate buffer and ammonium chloride. The dilution rates were varied by using culture vessels of different size. The culture was streaked at intervals of half a retention time on a nutrient supply agar medium. When the predominance of one colony type reached 90%, the organism was isolated and checked for purity and identity.

The mechanism for separation of one species from the other depends on the Maximum growth rate, μ_{max}, and the saturation constant, Ks, of the bacteria on a special substrate, as well as the dilution rate given in the chemostat system.

D.W. Tempest (42) has studied the mechanism of how a contaminant outgrows the original species. As he points out, if the contaminant has a higher μ_{max} value than the original species at the same growth substrate concentration, it will

replace the latter because of its ability to grow faster at same substrate concentration. The original species will be washed out because its μmax is lower and it thus cannot effectively compete for the substrate. (Fig. I)

The dependancy on μmax to select out one species from the other was shown by Jannasch in his enrichment studies. He combined a Pseudomonas sp. (with a μmax of $0.8 hr^{-1}$) and a Spirillum sp. (with a μmax of $0.6 hr^{-1}$) (obtained from separate chemostat enrichments) together in a chemostat system and set the dilution rate at 0.4 hr^{-1} and 0.7 hr^{-1}. He later isolated the Pseudomonas at a dilution rate of 0.7 hr^{-1} and Spirillum at a dilution rate of 0.4 hr^{-1} respectively, because at each dilution rate there is one bacterium grew faster than the other.

The use of continuous culture techniques in the classical sense to study hydrocarbon degradation is very limited because: (1) lots of hydrocarbon is required (2) the substrate must be continually and constantly dispersed (3) rubber tubing is required which will not be affected by hydrocarbons.

It has, however, been used with or success in several cases. Liu and Townsley (25) studied hydrocarbon degradation in continuous culture and found that the growth rate and acid production were dependent on the concentration of hydrocarbon, the concentration of nitrogen the presence of emulsifier. Moo-Young et al (31) also used a continuous culture system to study the n-dodecane fermentation by yeast Candida lypolytica. In their studies, it was found that the specific growth rate

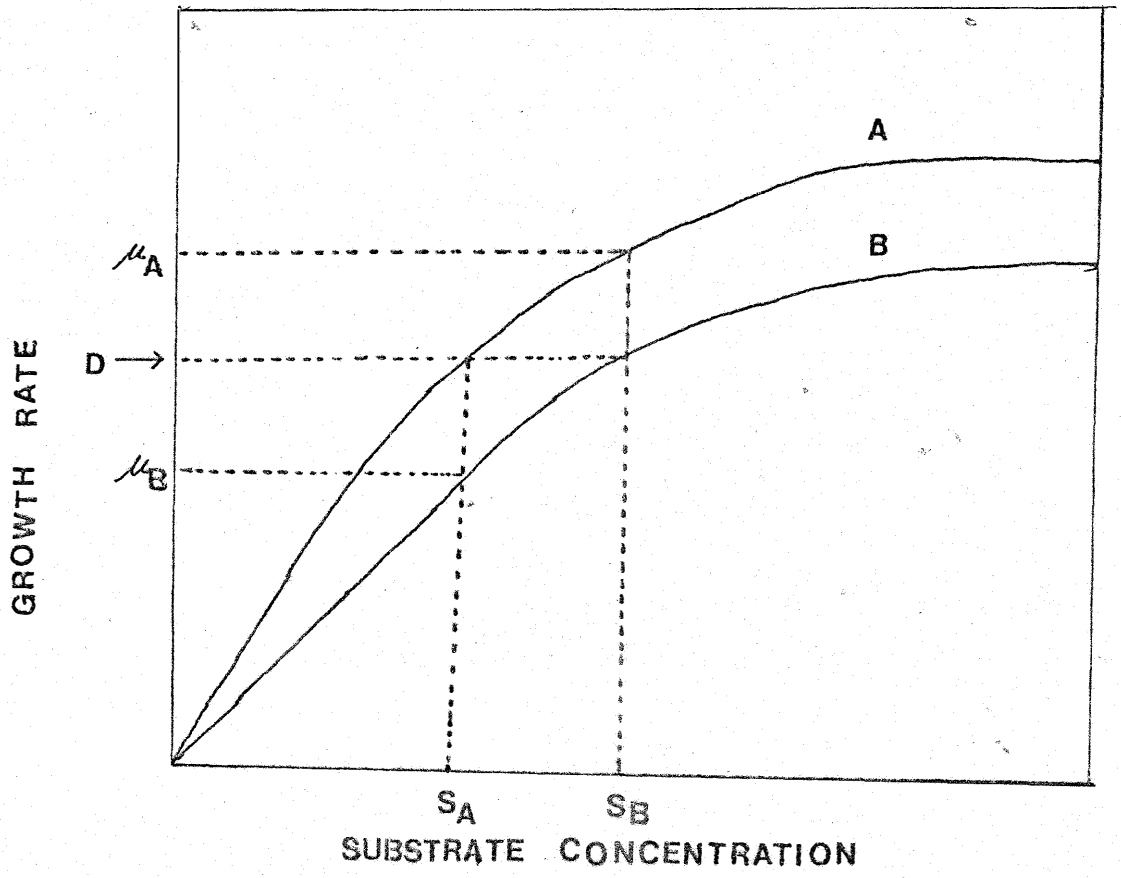


Fig.1. Theoretical saturation curves for two organisms (A and B) growing in separate chemostats in identical media. At a particular dilution rate(D), the growth-limiting substrate concentration would be S_A for organism A and S_B for organism B. If the two organisms were present in the same chemostat culture, operated at the same dilution rate, then organism A would outgrow B since at the growth-limiting substrate concentration S_A, organism B could only grow at the rate μ_B which, being less than D, would be insufficient to prevent it from being washed out of the culture(42).

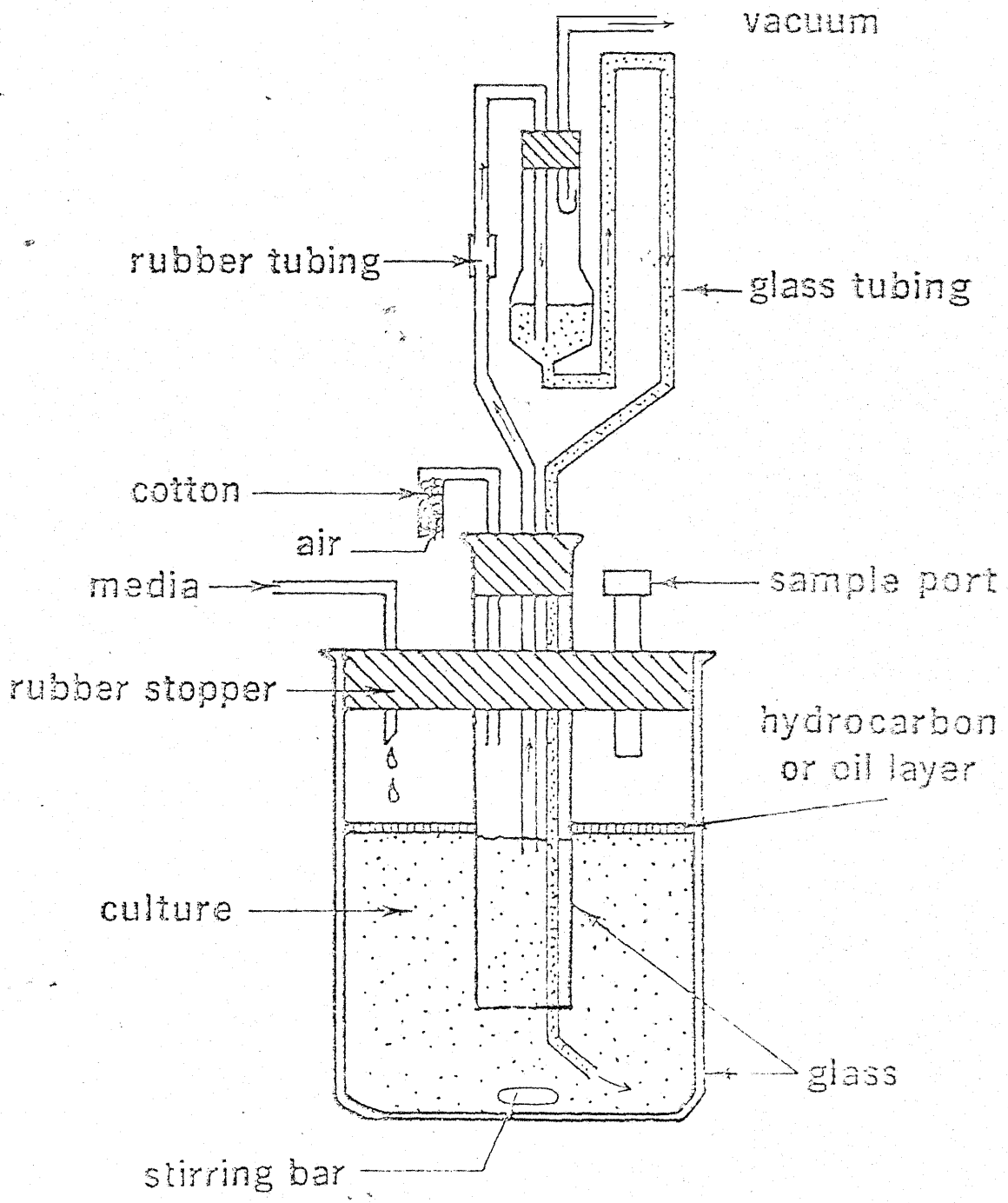
of cells was not only dependent on hydrocarbon concentration but also on size of dispersed oil droplets.

MATERIAL AND METHODS

a) Continuous culture apparatus : The continuous culture system contained a two layer system in a growth vessel, i.e., a hydrocarbon layer on the top of a water column. Water and nutrient solution were continuously supplied from a reservoir and a constant volume was maintained by an overflow device. To maintain sufficient oxygen concentration in the water phase, aliquots of the culture fluid were continuously cycled out of the growth vessel, aerated and recycled back into the growth vessel. For mixing, the aqueous phase was slowly stirred with a magnetic stirring bar without any emulsification of the floating hydrocarbon layer. The set up is illustrated in Fig. 2.

The flow of aqueous medium into the growth vessel (a 300 ml Berzelius beaker) was controlled by a peristaltic pump (Havard apparatus). All rubber stoppers were "Neogrene" brand. The aeration system was based on the use of a constant vacuum (water aspirator or mechanical pump) to suck air and culture medium up into an aerator tube (a 24x150 mm tube filter) above the culture vessel. The aerator tube was initially filled by pinching off the flexible tubing on the intake tubing so that liquid was sucked up into the aerator via the exit tubing. This procedure completed a siphon in the exit tubing. Then, once the intake tubing was reopened, liquid was continuously sucked up into the aerator and exited via this siphon. A reservoir of culture fluid (approximately 10 ml) was continuously maintained in the aerator tube by

Figure 2. Continuous Culture System.



virtue of constant vacuum applied.

b) Cultural conditions : For all experiments, octane was used as the sole carbon and energy source. The medium in the reservoir consisted of a basal salts formulation made up of NH_4Cl (100 mg/l), a phosphate buffer (KH_2PO_4 K_2HPO_4, 100 mg/l pH 7.2), $MgSO_4$ (10 mg/l) and distilled water. For mixed population studies, the growth vessel was inoculated with Lake Ontario water and for pure culture studies, M-2 (obtained from chemostat-enrichment) was used.

c) Batch culture enrichments : Each component of the basal salts medium was added to a 250 ml screw top Erlenmyer flask at a 10x concentration and the volume of the flask was brought up to volume (usually 100 ml) with a fresh sample of Lake Ontario water. Approximately 0.5 ml of filter sterilized octane was then added to the medium and the flask was incubated on a shaker. After the growth of cells for two or three days, 5.0 ml of cell suspension were transferred to a sterile medium containing 100 ml distilled water, basal salts and 0.5 ml octane. After turbidity appeared, a third transfer was made using 1 ml inoculum to the fresh medium. When the medium showed turbidity in the final transfer, the cell suspension was diluted and spread on plate count agar (Difco) plate and the most dominant species was isolated and purified. If there was no predominant species after the third transfer, a fourth transfer was made and isolation procedure was repeated until there was a predominant species.

d) Chemostat enrichments : An enrichment was set up

putting 220 ml of a fresh Lake Ontario water sample into a growth vessel and placing 8 ml sterile octane on the top of the water sample. The lake water inoculum was supplemented with basal salts at the concentration present in the reservoir. The growth vessel was then incubated as a batch culture for 15-24 hrs. before the dilution was started. The dilution rate was determined by the volume in the catch flask per unit time. The cell populations in the continuous culture system were assayed periodically using plate count agar (Difco). The most dominant species was isolated when it eventually occupied about ninty percent of the total population. To recheck the ability of isolates to utilize octane, the bacteria were streaked on mineral salts agar and incubated under an octane atomosphere. The appearance of colonies as compared to controls, indicated the use of octane as the sole source of carbon and energy.

e) Pure culture growth studies in continuous culture system : Inoculum for the continuous sulture system was prepared by harvesting 20-24 hrs. cells grown in shake flasks containing 0.1% sodium acetate and the basal salts medium. Cells were centrifuged at 1000 g for 10 minutes at 0^{o}c and washed twice with phosphate buffer (100 mg/l pH 7.2). The final turbidity of the resuspended cells was read in spectrophotometer (450 nm) and diluted with phosphate buffer to give an optical density of 0.3. In each pure culture study, the growth vessel was inoculated with 5 mls of this washed cell suspension.

f) Rate of Degradation : To monitor disappearance of octane, a fat soluble dye (Sudan III) was added to the octane layer at a concentration of 50 µg/ml. Since the octane would be removed into the aqueous phase, the dye would become more and more concentrated in the remaining octane layer. Thus, the rate of octane disappearance was measured as a function of the increase in optical density of dye solution. Samples (20 µl) to be used for absorbance measurement were taken directly from the octane layer using a capillary pipette (50 ul Pyrex disposable micropipette). They were then diluted 1:50 in hexane and read in a Colemn Junior II spectrophotometer at 510 nm. Rates were calculated using the modified Beer's equation:

$$A = EL\frac{D}{V_o - V_t\ T}$$

Where A=absorbance, E=extinction coefficient, L=the width of the cuvette, D=moles of dye, V_o=initial volume of hydrocarbon in liters, V_t= the volume of octane lost per unit time, and T=time. By taking the reciprocal of this equation, a graphical form was obtained in which a plot of $\frac{1}{A}$ against time gave a straight line. The volume of octane removed per unit time was calculated using the Y intercept, V_o/ELD and the slope V_t/ELD.

g) Identification and classification of isolates : For identification and classification, isolated bacteria were gram-stained, observed under a microscope, and characterized by a series of physiological tests. These tests are described as follows:

1) Oxidase test: A fresh, 1% solution of tetramethyl-p-phenylene diamine dihydrocholoride was prepared. A portion of a colony was removed with a thin glass rod and rubbed on a strip of filter paper impregnated with the oxidase reagent. The positive reaction was determined by the purple color formatiom on the bacteria deposits (6).

2) Hugh and Leifson test: The basal medium of Hugh and Leifson contatined: peptone 2g, NaCl 5g, K_2HPO_4 o.15g, KH_2PO_4 0.15g, agar 3g, distilled water 885 ml. The semi-solid agar was autoclaved and quickly cooled. Before the agar solidified, 100 ml of 10% (w/v) sterile solution of glucose and 15 ml of 0.2% sterile solution of Brom Thymol Blue (pH 7.3) were added to the basal medium. The medium was then tubed in 5 ml samples. Immediately before use these tubes were heated in a boiling water bath for ten minutes to drive off oxygen, cooled and inoculated by stabbing. One series of tubes were incubated aerobically (i.e. no precaution to exclude air) and another series were incubated anaerobically by sealing with vaseline. The cultures were observed at 7 and 28 days. The positive reaction for the utilization of glucose oxidatively or fermentatively was indicated by the color change form blue to yellow (12).

3) Indole production, gelatin hydrolysis, starch hydrolysis, MRVP test, nitrate reduction and simple fermentation tests were carried out using the standard media produced by Difco and BBL and following the standard procedures outlined in Collins and Lyne (6) and Benson (3).

4) To test for deamination of phenylalanine to phenyl pyruvic acid, cells were grown on a medium containing yeast extract 3 g., DL-phenylalanine 2 g., disodium phosphate 1 g., sodium chloride 5 g., Agar 12 g., and distilled water 1000 ml (7). The phenylalanine medium was prepared as an agar slant and incubated for 24 hrs. after inoculation. Following incubation, 4 or 5 drops of ferric chloride reagent (13% w/v, 0.5 M) were allowed to run down over the growth on the slant. The presence of pyruvic acid produced a green color in the medium.

5) Decarboxylation of arginine, ornithine and lysine were tested by detecting amine formation (i.e., increase in pH) on Difco decarboxylase media containing 1% concentration of each amino acid. The tubes of media without the addition of any amino acid were used as the control. The control media and the amino acid media were tubed in 3 or 4 ml amounts in small (13 x 100 mm) tubes and sterilized at 121°C for 10 minutes. The tubes were inoculated and overlaid with 4 to 5 mm of sterile mineral oil. The incubation was made at room temperature and examined daily for 4 days. Positive reactions were indicated by alkalinization of the media and a consequent change in the color of the indicator system from yellow to violet or reddish-violet. Weakly positive reactions were bluish in color (7).

6) To test for growth of isolated bacteria on carbon sources other than hydrocarbons, a basal salts medium was prepared containing glucose, Na acetate Na lactate or Na glutamate,

all at 0.1% concentration. The cell turbidity and growth character in each medium were used as criteria for utilization of these carbon sources.

RESULTS AND DISCUSSION

I. Enrichment Studies

The success of a continuous culture system in which the substrate was not dissolved in the aqueous phase depended on our ability to isolate bacteria which could degrade hydrocarbons at the hydrocarbon water interphase in the absence of significant mechanical dispersion.

In an attempt to isolate these bacteria, continuous culture enrichments were set up. Growth vessels of the continuous culture systems were inoculated with a mixed bacterial population using a Lake Ontario water sample. This was then supplemented with the appropriate inorganic salts and 10 ml of octane were placed on the surface of the water column.

The system was then incubated at room temperature as a batch culture for approximately 15 hours or until a slight turbidity was apparent.

At this time the flow of medium from the reservoir was started and changes in the mixed population were continuously followed by simple plate counting techniques.

A typical course of events is shown in Table 1. As can be seen, there were about five to six major colony types which originally appeared in the presence of octane. With continuous incubation, it can be seen that at about 10-12 retention volumes (336-360 hrs.), one of the original colonies became predominant eventually reaching 90% of the total population. This predominant species persisted at this level

TABLE 1

APPEARANCE OF BACTERIAL COLONY TYPES DURING A CONTINUOUS CULTURE ENRICHMENT STUDY AT DILUTION RATE OF 0.03 HR^{-1}

Time	Bacteria (% of Total Population)					
Hours	M_1	M_2	M_3	M_4	M_5	M_6
0	24	22	15	27	9	3
96	10	42	5	25	15	3
168	5	70	0	11	14	0
360	2	88	2	8	0	0
432	0	90	2	8	0	0
529	0	90	0	10	0	0
600	0	90	0	10	0	0

through out the rest of the experiment.

The selection of one dominant species from a mixed population by a chemostat enrichment agrees with the work of Jannasch (16) who was using simple soluble sugars and organic acids as substrates. The selected species is believed to be the fastest octane utilizer under the conditions employed.

Changes in the population densities and the rate of octane removal also reflected the enrichment process. As can be seen in Figure 3 the total population density rose slowly to a value of 8.0×10^9 cells/ml and then leveled off in an apparent steady state. Likewise, once the predominant species appeared, it too increased to a steady state level and in fact eventually represented almost the entire total population density. Other enrichment experiments have shown similar population density changes which always resulted in a steady state condition. In most experiments, the predominant species (i.e. 90% of total population) appeared after the first 11 retention volumes.

The rate of octane removal was determined by the Sudan III dye method described in Materials and Methods. As can be seen in Figure 3, significant removal of octane did not occur until about 10 retention volumes (336 hr) at which time it commenced at a linear rate of 13.0 mg/hr. In this experiment as well as in other enrichment, the onset of octane removal correlated with the appearance of the predominant species. Thus, it appeared that a single bacterial species was more efficient at removing octane than was the mixed-

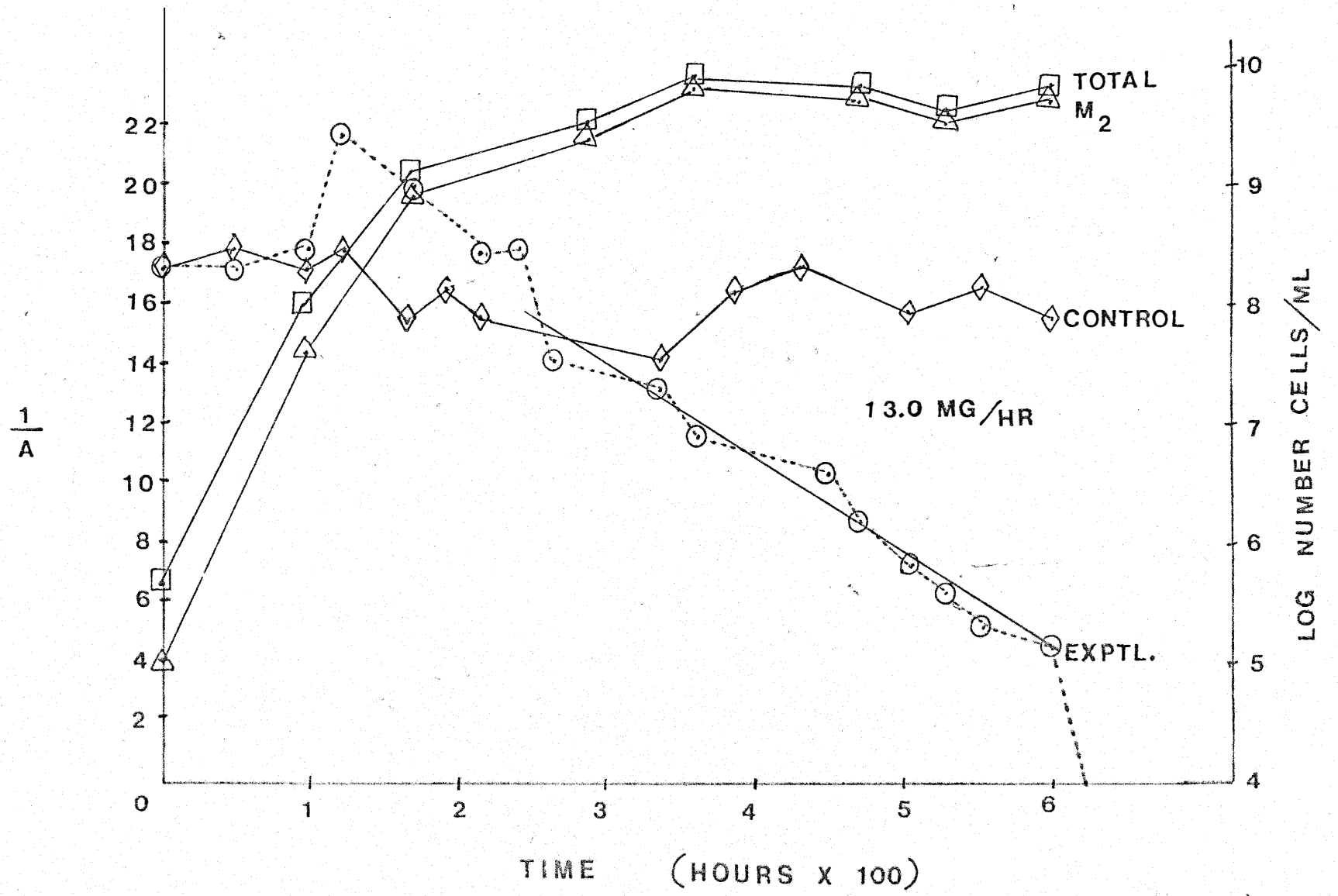


Fig. 3. The Rate of Octane Removal (as a Function of Sudan III Concentration in Hydrocarbon Layer) and Changes in Cell Density (Based on Colony Counts) During a Dilution Rate of 0.03 hr^{-1}. Total Cell Concentration, ⊡ ; Predominant Species, M_2, △ ; Rate of Octane Removal, ⊙ ; Control (No Bacteria), ◇ .

population or as a predominant species, was needed to get significant hydrocarbon removal.

In all experiments run so far the rate of octane removal has been constant with time, i.e. producing a straight-line relationship when the reciprocal of absorbance was plotted against time. This constant rate of removal was not a function of solubility or evaporation since control experiments in the absence of bacteria did not show any octane removal. The only deviation seen from this constant rate of removal was in several enrichment experiments in which, toward the end of the experiment when there was only a small amount of octane remaining in the hydrocarbon layer, a sudden increase in removal occurred. (Figure 3). This increase always occurred when very little octane remained and has so far only been seen with mixed culture experiments. It is difficult at this time to tell whether the rate increase was due to a physical artifact of the continuous culture system or to some emulsification phenomena brought on by the growth of the bacteria.

Jannasch's work with the chemostat enrichment process using soluble substrates showed that a simple change of dilution rate or of substrate concentration in the reservoir also changed the enrichment pattern in that an entirely different bacterial species was selected out. In an attempt to determine whether a similar process could occur in our continuous culture systems using an insoluble substrate, enrichment experiments were carried out using different

dilution rates. Table 2 shows the results of two enrichment experiments run at dilution rates of 0.03 hr^{-1} and 0.06hr^{-1} using the same inoculum for each growth vessel and keeping other conditions as constant as possible.

As can be seen from the table, the rate of octane removal at dilution rate of 0.06hr^{-1} was about 2½ fold faster than at dilution rate of 0.03hr^{-1}, although about the same lag was required for two enrichment experiments. As expected, two different bacteria named M_1 and M_2 were selected out. Both M_1 and M_2 have been proved to utilize octane by growth on mineral salt agar plate under an octane atomosphere. Microscopic observation, colony morphology examination, and a series of biochemical tests has indicated that they are different bacterial types. The different enrichments obtained without changing of substrate concentration, was apparently dependent on the dilution rate employed. It was also a reflection of their differential ability to attack the insoluble substrate at the hydrocarbon-water interface.

Successful enrichments have also been obtained at dilution rates of 0.1 hr^{-1} and 0.2 hr^{-1}. Rates of octane removal were faster being 42mg/hr and 51.8mg/hr respectively. However, these results were tentative since some of the octane may have been lost due to the use of black rubber stoppers. If in fact the rates of octane removal were higher at higher dilution rates, then it agrees with general chemostat theory. This must mean then, that the ability to attack a nondispersed hydrocarbon was a function of some minimal contact time with

TABLE 2

COMPARISON OF ENRICHMENT STUDIES AT DIFFERENT DILUTION RATE

	Dilution Rate	
	0.03 hr^{-1}	0.06 hr^{-1}
Rate of Octane Removal (mg/hr)	13.0	32.6
Lag* (Retention Volume)	10.0	11.5
Predominant Species	M_2	M_1
Characteristics:		
Gram Stain	-	-
Cell Morphology	Rod	Rod
Colony Morphology#	Circular Convex Smooth Glistening	Filamentous Convex Smooth Glistening
Pigment (Whole Colony)	Yellow Green	Yellow Green
Oxidase Test	+	+
HUGH & LEIFSON Test		
Oxidative	-	-
Fermentative	-	-
Indole Production	-	-
Gelatin Hydrolysis	-	-
Starch Hydrolysis	+	+
M R Test	-	-
V P Test	-	-
Nitrate Reduction#	+	-

TABLE 2 (Continued)

COMPARISON OF ENRICHMENT STUDIES AT DIFFERENT DILUTION RATE

	Dilution Rate	
	0.03 hr^{-1}	0.06 hr^{-1}
Glucose Fermentation	-	-
Lactose Fermentation	-	-
Phenylalanine Deamylase	-	-
Arginine Decarboxylase	+	+
Ornithine Decarboxylase	-	-
Lysine Decarboxylase	-	-
Growth on 0.1 % Glucose	-	-
Growth on 0.1 % Na-Acetate	+	+
Growth on 0.1 % Na-Glutamate	+	+
Growth on 0.1 % Na-Lactate	+	+

* Period of time when no octane removal was detected; measured from time of inoculum until octane removal commenced.

Only different characteristics between M_1 and M_2.

the hydrocarbon layer which was an inherent characteristic of the species enriched.

It should be mentioned that during a continuous culture enrichment, cell clumps was sometimes found on the wall of growth vessel, the end of glassware, as well as inside of the rubber tubings. The wall growth was a characteristic found only in mixed population studies, especially during the pre-incubation period as a batch culture when the cell turbidity was high. Such growth persisted at low levels throughout the experiments but was thought to have no significant effect on the enrichment process. It is more likely that it represented background growth which because of its adherence to the wall, prevented it from being washed out. In many cases a rubber scraper was used to remove the bacteria from the walls and this had no effect on the enrichment process or the steady state population.

In each mixed population study, one species was always selected out. Many other species which were originally present in mixed population either disappeared or became minor parts of the total population. It is clear that these enriched bacteria competed for hydrocarbon as carbon sources and were not affected by different environmental conditions since these were kept relatively constant.

Since the bacteria were growing in a water medium with the hydrocarbon floating in a non-dispersed state, it is probable that the bacteria selected out were specifically adapted to attacking the hydrocarbon in a non-dispersed state. Obser-

vation of cells microscopically indicated no internal deposits of hydrocarbon droplets and showed no hydrocarbon droplets associated with the surface of cell. Thus the method of attack appeared to be somewhat unique. In nature these bacteria which were revealed by the continuous culture enrichment probably complement the metabolic activities of bacteria which attack only dispersed hydrocarbons.

The enrichment experiments strongly suggested that the mechanism of octane removal was through bacterial assimilation and oxidation. The observation of an enrichment phenomenon and the ability to maintain a steady state bacteria population both support the contention that growth was taking place at the expense of some carbon and energy source; namely the utilization of octane. The abundance of evidence in the literature for the terminal oxidation of normal alkanes to their corresponding fatty acids supports a similar oxidative attack in our continuous culture systems. This question of actual degradation will be discussed further in the pure culture studies section.

II. Pure culture studies

A) Rate of Octane Removal by Isolate M_2

The success of the continuous culture enrichment experiments allowed an organism to be isolated which was known to grow and utilize octane in our continuous culture systems. With this isolate it was possible to determine some of the conditions which affected the octane removal with the idea that some knowledge could be gained as to the mechanisms

involved in this process. Ultimately we wanted to find out what particular characteristics were necessary for the bacterial attack of a nondispersed hydrocarbon layer.

Isolate M_2, which was obtained from a continuous culture enrichment was purified and then inoculated into a sterile continuous culture system. The rate at which it removed octane was followed by the dye method. A typical result is shown in Figure 4. As can be seen, the rate of octane removal was 11.0 mg/l. Some variation in the rates was however, observed generally within $\pm$ 4 mg/hr. Without knowing the exact way in which bacteria attack and assimilate the hydrocarbon layer, one would assume that the rates of removal would be very similar from one experiment to the next. However, several problems were involved in obtaining identical rate due to the physical nature of the experimental system. Octane was considerably more volatile than originally anticipated and consequently it was necessary to have a leak-free system. This evaporation loss was difficult to monitor especially since the system had to be repeatedly opened for sampling. Control experiments, however, not using bacteria, indicated that if one is careful, relatively little evaporation took place. But, since each experiment itself required two or three weeks of incubation and because it was difficult to run more than two continuous culture vessels at a time, it was often impractical to run a control with ever experiment. Ideally an internal control is more desirable, such as measuring bacterial degradation products (i.e. fatty

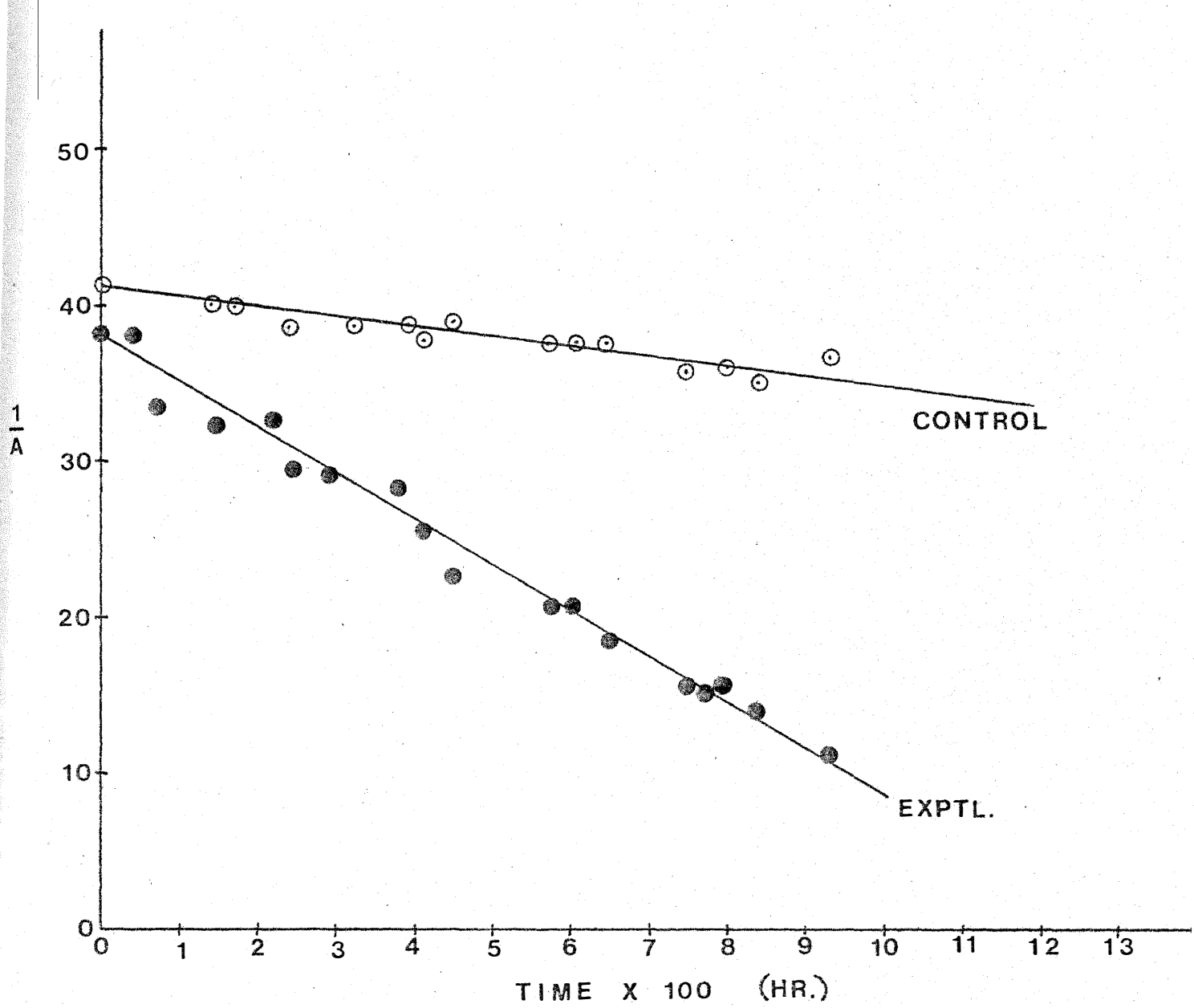


Fig. 4. Rate of Octane Removal by Isolate M_2 at Dilution Rate of 0.05 h

acids) because this would differentiate between evaporation and actual degradation. Thus some of the inability to obtain consistent rates was due to evaporation and other physical factors and their quantitative effects could not be determined at this time.

There is also some possibility that the presence of rubber stoppers and tubing could affect the rates. A number of experiments had to be discarded because of absorption of the hydrocarbon by a black rubber stopper. All of the continuous culture systems now have "Neogrene" stoppers which are considerably more resistant to the hydrocarbons.

The Sudan III dye method for observing removal rates has worked out very well and it is unlikely to have been the cause of the variation in rates. It has proven to be a very reliable and sensitive method which was easily standardized. The removal rates were not a function of the loss of Sudan dye since after a period of incubation the amount of octane lost could be added back (without dye) and the original optical density obtained. Its only disadvantage is that it is not a direct measure of hydrocarbon degradation by bacteria.

Because of the nature of a continuous culture system, one would also expect that factors like inoculum size, cell age and pre-incubation with the substrate would not affect the ultimate hydrocarbon removal rate. However, the inability to get the same removal rate using M_2 in pure culture suggested that these factors were important and therefore should be standardized. In this regard several experiments were set up

to test their effect on the removal rates.

B) Evidence for Actual Octane Degradation

The rate of removal of octane from the hydrocarbon layer using the dye technique did not directly demonstrate actual degradation of hydrocarbon by the bacterial population present, but it was highly indicative of such a process. Other pieces of evidence, however, did further verify a true degradation process.

The growth of bacteria and the maintenance of the steady state population througout continuous culture experiment indicated that the bacteria were using octane as sole carbon and energy source, since the octane was the only carbon source available. Experiment have also been done in batch culture which have shown that no growth occurred in flask without octane. Furthermore, if ammonium salts were eliminated from the inflowing medium the cells washed out of the growth vessel and the octane remained. Production of fatty acids from the oxidation of octane also has been shown in our laboratory by gas chromatography analysis (37). The dependence on oxygen during octane removal was also observed by the slow degradation rate of octane when aeration stopped.

C) Dependence of Octane Removal on inoculum Size

For detection of the effect of cell density on the degradation rate of octane in continuous culture, experiments were set up in which one growth vessel was inoculated with 5 ml (1x) and the other 15 ml(3x) of a washed cell suspension.

at an optical density of 0.3.

The rate of octane removal is shown in Fig.5. As can be seen, the rate of removal with the 3x cell inoculum was about two fold faster than 1x cells and persisted throughout the experiment.

Cell assay during experimental period showed that although the initial inoculum size was different, the cell population in both systems reached the same steady state concentration (Table 3).

This situation has been observed several times and the results are difficult to explain. One would expect that higher cell densities might increase octane removal (i.e. it might increase the production of an emulsifier of some kind), but under the conditions of these experiments the higher population density would be transient occuring only at the early phase of incubation. The cell numbers in fact showed this to be the case (see Table 3). It would then be expected that octane removal would also decrease from an initial high level to a level equivalent to that seen for the 1x cell inoculation. Since this was not the case, it is possible that the higher population density induced some phenomenon which allowed the cells to remove the octane at a higher rate but that the induction phenomenon remained even though the cell density decreased. Such an induction process could perhaps be associated with the production of an emulsifier or some mechanism of hydrocarbon transport.

D) Effect of Pre-incubation on Rate of Octane Removal

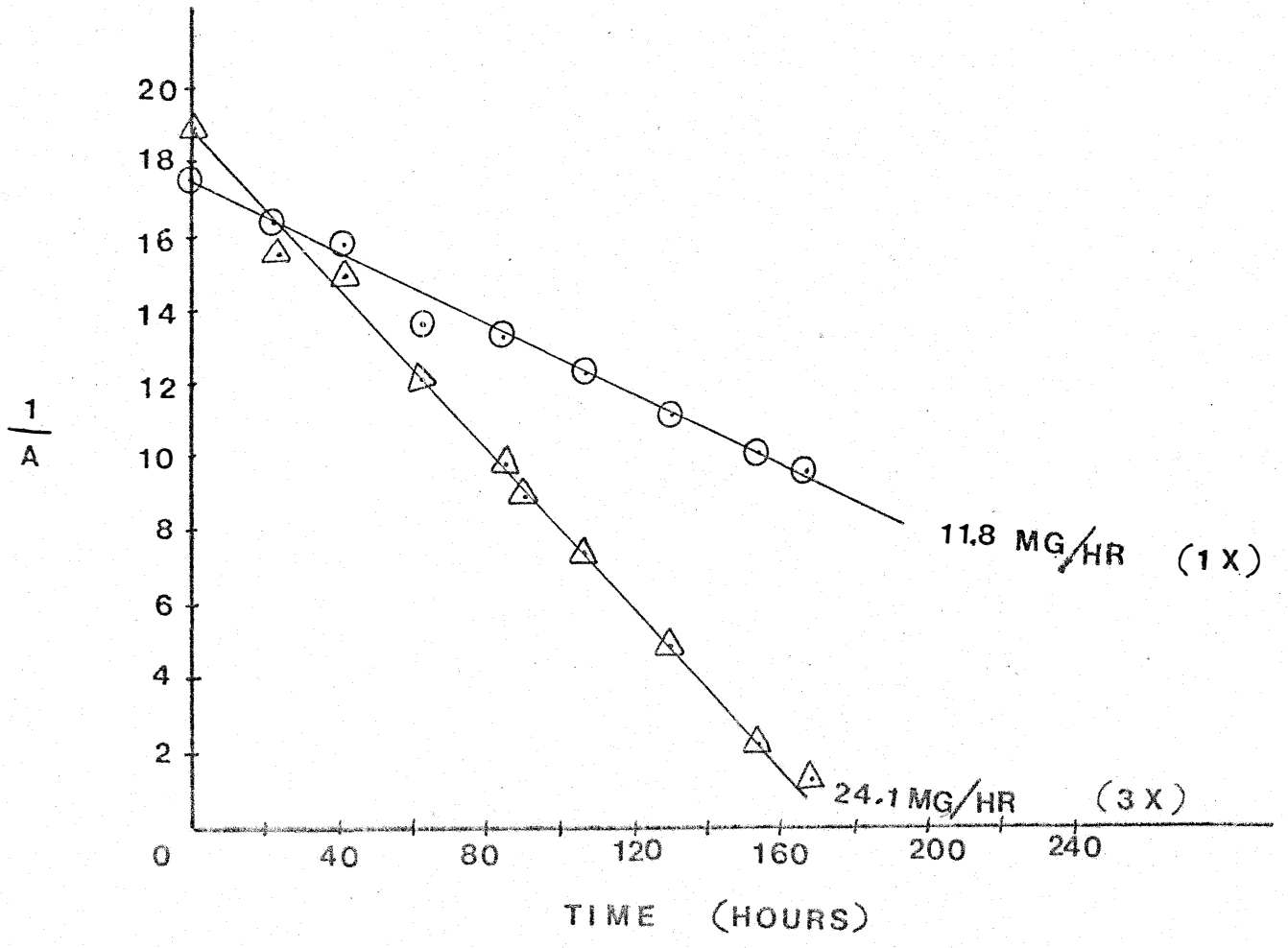


Fig. 5. The Effect of Inoculum Size on the Rate of Octane Removal by Isolate M_2 at a Dilution Rate of 0.05 hr^{-1}. 1X and 3X Indicate a Three Fold Difference in in Inoculum Size.

TABLE 3

CHANGES IN POPULATION OF TWO CONTINUOUS CULTURE SYSTEMS RUN UNDER IDENTICAL CONDITIONS EXCEPT FOR INOCULUM SIZE. 1X AND 3X INDICATE A THREE FOLD DIFFERENCE IN INOCULUM SIZE. Dilution Rate = 0.05 HR^{-1}.

Time (Hours)	1X ($X10^7$) Cells/ml	3X ($X10^7$) Cells/ml
0	6.8	16.0
11	3.5	6.5
39	1.0	5.5
60	2.0	2.3
89	1.1	2.5
129	2.2	1.8

In this study, 5 ml of the standard washed cell suspension (OD = ●.3) was inoculated to each of three continuous culture vessels, and incubated as batch cultures (i.e., D = $0hr^{-1}$) for o hr, 24 hrs and 48 hrs respectively. After the period of preincubation, the flow rate was started (D = $0.05hr^{-1}$), so that the cells were incubated under continuous culture conditions. The rates of octane removal are shown in Fig. 6.

Pre-incubation apparently did not have a significant effect on the removal rates. There was a slight difference in the rate after 48hrs of preincubation as a batch culture, but the difference is probably not above experimental error. It should also be noted in these experiments that during the preincubation period the rate of octane removal was considerably faster than under continuous flow conditions. No increased mechanical agitation occured during this time of preincubation and there was no other obvious factor which might have induced the higher rates. Some increase of turbidity was noted in the growth vessels after the preincubation period but this apparently had no effect on the subsequent removal rates. Cell densities in fact at 70 hours into the continuous culture incubation were $2\text{-}3x10^{8}$ cells/ml indicating that whatever initial differences there was in cell densities, they rapidly readjusted themselves to coincide with the continuous culture growth conditions.

E) Effect of Dilution Rate on the Rate of Octane Removal

According to continuous culture theory, cells growing in a chemostat have a rather wide range of dilution

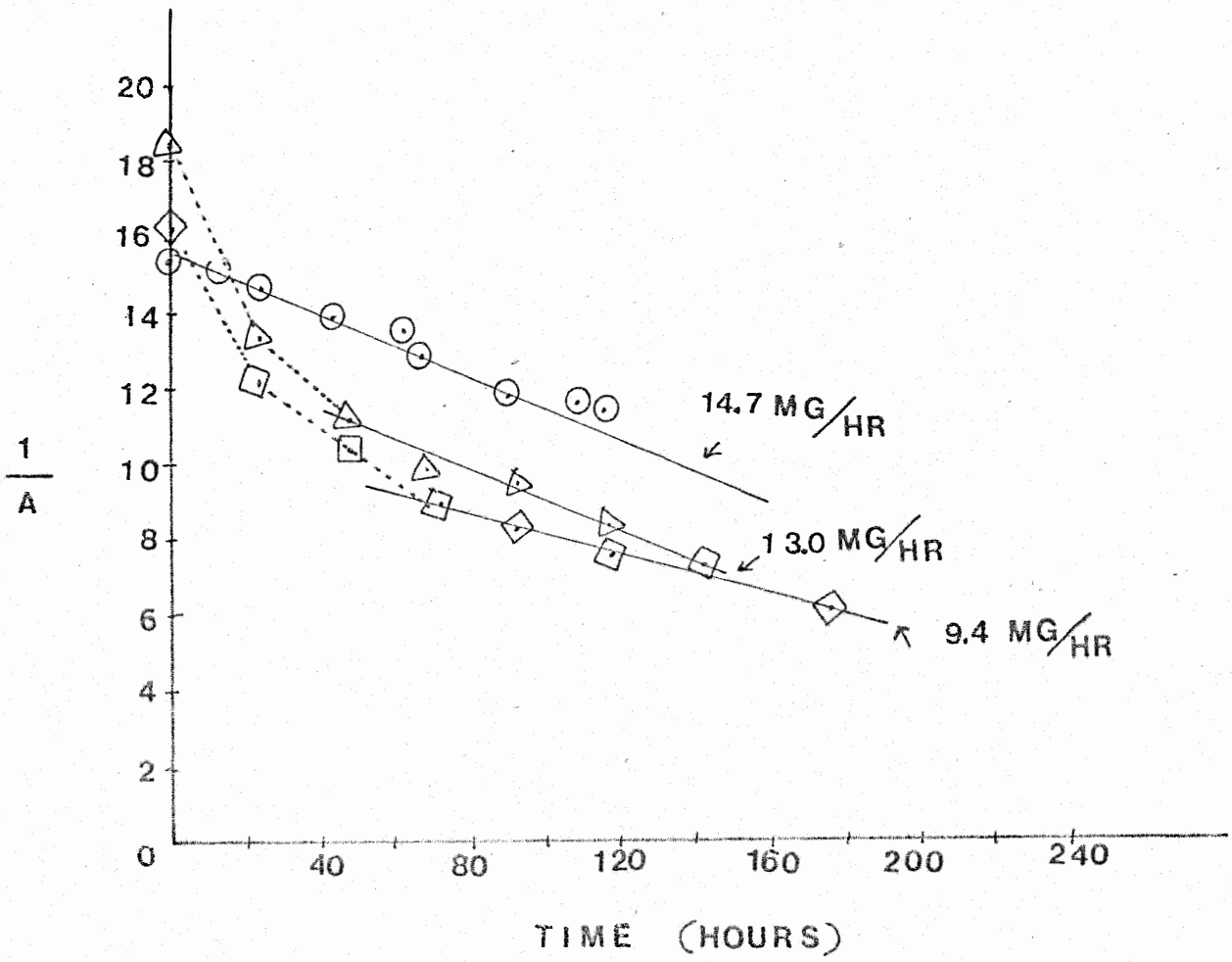


Fig. 6. Effect of Preincubation as a Batch Culture ($D=0\ hr^{-1}$) on the Rate of Octane Removal under Continuous Culture Conditions ($D=0.05\ hr^{-1}$) by Isolate M_2. Dashed Line Indicates Duration of Preincubation. No Preincubation, ⊙ ; 24 hr Preincubation, ▲ ; 48 hr Preincubation, ⊡ .

rates at which they will grow without being washed out. If the concentration in the reservoir is kept the same, an increase in the dilution rate will not significantly affect the steady state population density but will increase the rate of substrate consumption since the steady state substrate concentration (unconsumed substrate in growth vessel) also remains relatively constant.

In our continuous culture system, where the substrate floats on the surface of the aqueous phase, one would expect one of two possibilities; either a) hydrocarbon is removed at a faster rate as dilution rate is increased or b) hydrocarbon removal decreases due to inability of the cells to attack the hydrocarbon because of decreased time of contact.

Experiments, however, which tested the effect of dilution rate on the rate of octane removal have shown that the rate was not affected by the change in dilution rate (Fig. 7). Instead the steady state population density decreased at the higher dilution rates (Table 4). Thus, it appeared that at higher dilution rates, the same amount of octane was removed by a small number of cells. This would mean that the faster growth rate of M_2 caused a faster rate of octane removal. However, the amount of octane available to the steady state population must have been less because the population density was lower.

From these experiments we can conclude that standardization of the experimental conditions was necessary in order to obtain a constant rate of octane removal. Under the standard

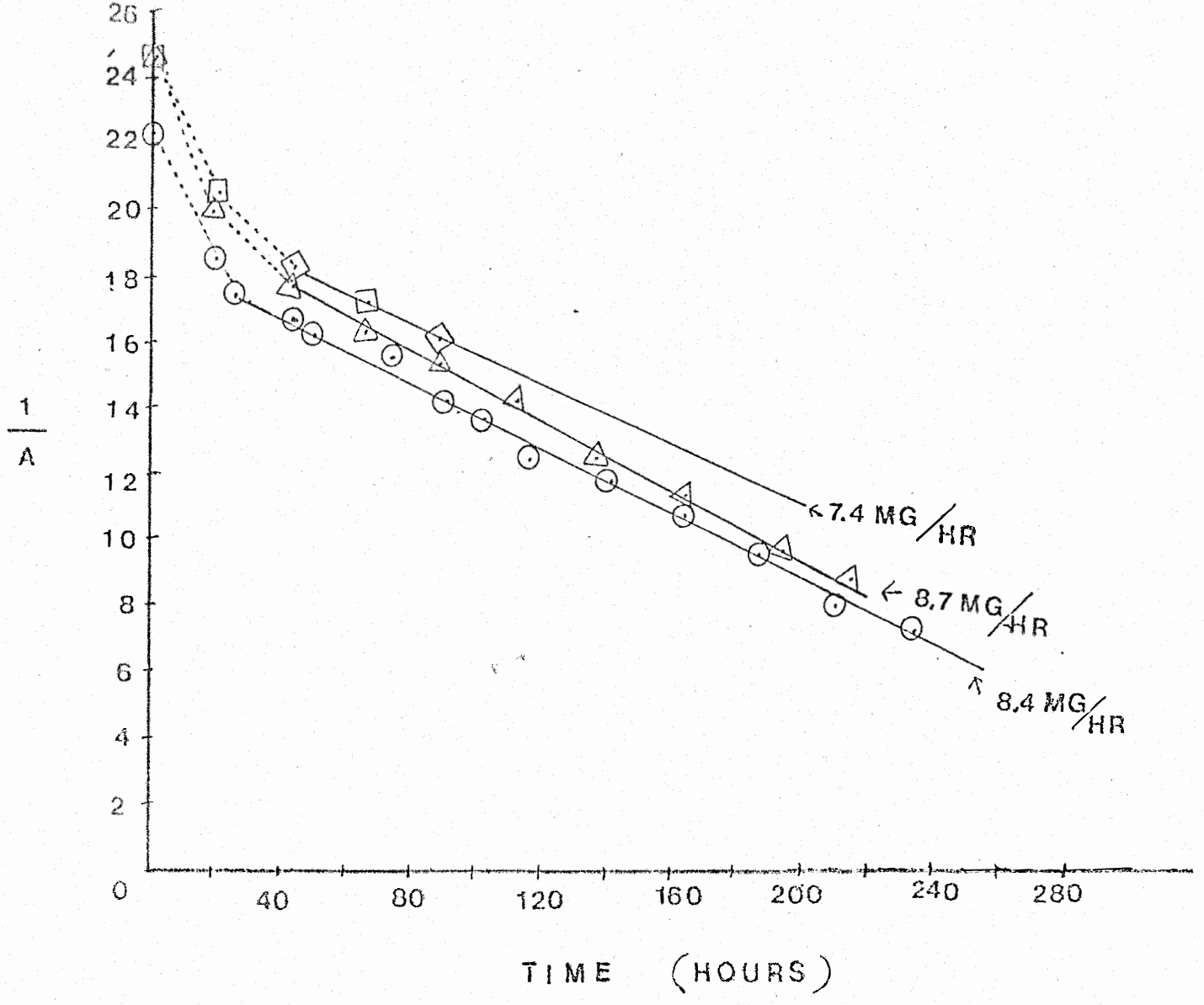


Fig. 7. Effect of Dilution Rate on Octane Removal Using Isolate M_2.
$D=0.05\ hr^{-1}$, ⊙ ; $D=0.2\ hr^{-1}$, △ ; $D=0.4\ hr^{-1}$, ⊡ .

TABLE 4

DIFFERENCES IN STEADY STATE POPULATION DENSITIES DURING OCTANE REMOVAL AT DIFFERENT DILUTION RATES USING ISOLATE M_2

Time	Cell Numbers Per Ml ($X10^6$)		
Hours	$D=0.05\ hr^{-1}$	$D=0.20\ hr^{-1}$	$D=0.40\ hr^{-1}$
0	500	150	150
20	450	2	5
43	200	—	—
66	—	1	—
89	—	4	4
91	100	—	—
113	—	—	—
117	400	7	—
187	200	—	—

conditions, the rate of removal averaged around 11 mg/hr which was very close to the removal rate observed in the original continuous culture enrichment of M_2.

F) Possible Mechanisms by which Cell Attack Octane

The solubility of octane in water at 25°c as indicated by Johnson (18) is only about 6.6×10^{-1} mg/l. Since the degradation rate obtained in our studies far exceeded this solubility, it was possible that bacteria attack via some other means. Johnson (18) expressed a hypothesis that a micro-organism was able to attack a hydrocarbon droplet and transfer it from outside of the cell membrane to inside of the cell where the enzymes responsible for primary oxidation exist. It is still doubtful at the present time whether direct contact between hydrocarbon and micro-organism is necessary in hydrocarbon degradation. However, Muck et al (32) in their experiments have shown that hydrocarbons penetrated into the cell wall and accumulated on the cytoplasmic membrane of the yeast Candida lypolytica. The association with the membrane caused noticeable ultrastructure changes such as the increase of the cytoplasmic membrane surface and a presence of higher amounts of mitochondria. Radioisotope studies also indicated that labelled hydrocarbon entered and left the cell within 60 sec. When they removed cell wall, however, they found no hydrocarbon uptake or oxidation. A method of direct attack on the hydrocarbon by cells may be true in a mechanically dispersed state but is difficult to apply it to our oil-water two layer system.

Another possible mechanism for the utilization of insoluble hydrocarbons is production of emulsifiers. The excretion of emulsifing factors into the growth medium has been found in the yeast Candida and the bacterium Pseudomonas when grown on hexadecane (13). These factors were thought to increase the rate or reduce the lag time in the microbial degradation of oil pollutants. There is at present no direct evidence for the production of emulsifiers in our continuous culture systems since octane was always removed at a constant rate.

In our studies, two conditions are important to note since they reflect on the ability of the organism to attack the hydrocarbon. First, in all experiments M_2 always grew in the dispersed state; cells did not accumulate under the hydrocarbon layer. It is not clear whether the cells are attacking the hydrocarbon in the dissolved state or in the mini-droplet state. Second, the hydrocarbon layer remained completely intact and there were no droplets of hydrocarbon observed under the microscope. It is possible that the bacteria studied were producing a continuous amount of emulsifing agent. This idea is supported by the fact that if large number of cells were present, the rate of octane degradation were increased.

CONCLUSIONS

From the above studies, the following conclusions can be made:

a.) The degradation of hydrocarbons by bacteria does not require mechanical or chemical dispersion of the substrate. Bacterial species can be readily obtained which will attack a stable non-dispersed hydrocarbon layer floating on the surface of a water column.

b.) Continuous cluture techniques can be used to study hydrocarbon degradation and the particular system employed in this study is entirely adequate for such studies.

c.) Continuous culture enrichments are also possible with this type of system. The selecting out of one particular bacterial species means that the bacteria compete for substrate even though it is not uniformly dispersed throughout the culture medium.

d.) The incorporation of Sudan III dye into the octane layer to measure the rate of octane degradation was very reliable, accurate and simple. It suffers from the fact that it is not a direct measure of octane degradation by bacteria.

e.) The degradation of octane by mixed populations of bacteria does apparently commence at a significant rate until a particular species has become predominant.

f.) The degradation rate of octane by a pure culture, M_2, is relatively constant from one experiment to the next indicating that the growth of M_2 is a function of substrate

availability and not a function of some artifact inherent in the continuous culture system itself.

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APPENDIX A

DATA FOR STANDARD CURVE

Dye Concentration (mg Sudan III / 100 ml Octane)	% Transmittance	Relative Optical Density
2.5	92	0.036
5*	86	0.065
8.25	77.5	0.11
10	75	0.125
12.5	74	0.132
16.5	64	0.15
25	52.5	0.28
37.5	44.5	0.352
50	33	0.482
62.5	29	0.54
75	21	0.68
87.5	19	0.72
100	15	0.83
125	11	0.96
150	9	1.05
200	7	1.15
250	6	1.23

* 5 mg/100 ml is the concentration used in chemostat.

APPENDIX B

APPEARANCE OF BACTERIAL COLONY TYPES DURING CONTINUOUS CULTURE ENRICHMENT STUDIES AT DIFFERENT DILUTION RATES

Name of Isolates	Dilution Rate (hr^{-1})	Time of Experiment (hr)	% of Total Population
M_1	0.06	210	88
M_2			0
M_3			0
M_4			12
M_5			0
M_6			0
M_7	0.05	240	10
M_8			70
M_9			15
M_{10}			5
M_{11}	0.2	120	2
M_{12}			4
M_{13}			94
M_{14}	0.1	120	90
M_{15}			7
M_{16}			3

APPENDIX C

CHARACTERISTICS OF ISOLATES

	NAME OF ISOLATES				
	M_3	M_4	M_5	M_6	M_7
Gram Stain	-	-	+	+	-
Cell Morphology	rod	short rod	rod	rod	short rod
Colony Morphology	circular pulvinate smooth glistening	circular pulvinate smooth glist.	filamentous pulvinate smooth glist.	circular flat rough	circular pulvinate smooth glist.
Pigment (Whole Colony)	yellow green	yellow green	white	white	white
Pigment (Plate)	___	___	___	Blue	___
Oxidase Test	+	+	+	+	+
HUGH & LEIFSON Test					
Oxidative	-	+	+	+	+
Fermentative	-	+	-	-	+
Indole Production	-	-	-	-	-
Gelatin Hydrolysis	-	-	+	+	+
Starch Hydrolysis	+	+	+	+	+

APPENDIX C

CHARACTERISTICS OF ISOLATES (Continued)

	NAME OF ISOLATES				
	M_3	M_4	M_5	M_6	M_7
M R Test	-	-	-	-	-
V P Test	-	-	-	-	-
Nitrate Reduction	+	+	-	+	-
Glucose Fermentation	-	-	-	-	-
Lactose Fermentation	-	-	-	-	-
Phenylalanine Deamylase	-	-	-	-	-
Arginine Decarboxylase	+	-	+	+	+
Ornithine Decarboxylase	-	-	-	-	-
Lysine Decarboxylase	-	-	-	-	-
Growth on 0.1 % Glucose	-	+++	+++	+++	+
Growth on 0.1 % Na-Acetate	+	+	-	+	+
Growth on 0.1 % Na-Glutamate	+	++	+	++	+
Growth on 0.1 % Na-Lactate	++	+	+++	+++	+

APPENDIX C

CHARACTERISTICS OF ISOLATES (Continued)

	NAME OF ISOLATES				
	M_8	M_9	M_{10}	M_{11}	M_{12}
Gram Stain	-	-	-	-	-
Cell Morphology	short rod	short rod	rod	long rod	rod
Colony Morphology	filamentous pulvinate smooth glistening	filamentous pulvinate smooth glist.	irregular flate rough	irregular flat rough	circular convex smooth glist.
Pigment (Whole Colony)	white	white	yellow green	white	yellow green
Pigment (Plate)	___	___	___	___	___
Oxidase Test	+	+	+	+	+
HUGH & LEIFSON Test					
Oxidative	+	+	-	+	+
Fermentative	+	+	-	+	-
Indole Production	-	-	-	-	-
Gelatin Hydrolysis	+	+	-	-	-
Starch Hydrolysis	+	+	+	+	+

APPENDIX C

CHARACTERISTICS OF ISOLATES (Continued)

	NAME OF ISOLATES				
	M_8	M_9	M_{10}	M_{11}	M_{12}
M R Test	-	-	-	-	-
V P Test	-	-	-	-	-
Nitrate Reduction	-	-	-	-	+
Glucose Fermentation	-	-	-	-	-
Lactose Fermentation	-	-	-	+ (acid only)	-
Phenylalanine Deamylase	-	-	-	-	-
Arginine Decarboxylase	+	+	+	-	+
Ornithine Decarboxylase	-	-	-	-	-
Lysine Decarboxylase	-	-	+	-	-
Growth on 0.1 % Glucose	+	++	-	+	-
Growth on 0.1 % Na-Acetate	++	+	-	-	±
Growth on 0.1 % Na-Glutamate	++	++	+	-	+
Growth on 0.1 % Na-Lactate	++	+	-	-	+

APPENDIX C

CHARACTERISTICS OF ISOLATES (Continued)

	NAME OF ISOLATES			
	M_{13}	M_{14}	M_{15}	M_{16}
Gram Stain	-	-	-	-
Cell Morphology	short rod	rod	rod	short rod
Colony Morphology	circular convex smooth glistening	circular convex smooth glistenign	irregular convex rough	irregular flat rough
Pigment (Whole Colony)	white	yellow green	yellow green	yellow green
Pigment (Plate)	tan	___	___	blue
Oxidase Test	+	+	+	+
HUGH & LEIFSON Test				
Oxidative	+	-	-	-
Fermentative	-	-	-	-
Indole Production	-	-	-	-
Gelatin Hydrolysis	+	-	-	-
Starch Hydrolysis	+	+	+	+

APPENDIX C

CHARACTERISTICS OF ISOLATES (Continued)

	NAME OF ISOLATES			
	M_{13}	M_{14}	M_{15}	M_{16}
M R Test	-	-	-	-
V P Test	-	-	-	-
Nitrate Reduction	-	+	+	-
Glucose Fermentation	-	-	-	-
Lactose Fermentation	-	-	-	-
Phenylalanine Deamylase	-	-	-	-
Arginine Decarboxylase	+	+	-	+
Ornithine Decarboxylase	-	-	-	-
Lysine Decarboxylase	-	-	-	-
Growth on 0.1 % Glucose	++	-	$\pm$	-
Growth on 0.1 % Na-Acetate	++	$\pm$	+	-
Growth on 0.1 % Na-Glutamate	++	+	+	+
Growth on 0.1 % Na-Lactate	++	+	+	+